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The Influence of L-PRF in Socket Healing with Immediate Implants: Proposal of a Prospective Randomized Split-mouth Study Design

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À minha Mãe, o meu maior apoio e

À minha Avó, que a levo comigo no coração…

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"It always seems impossible until it's done"

Nelson Mandela

List of abbreviations

A-PRF	Advanced Platelet-Rich Fibrin
A-PRF^{+}	Advanced Platelet-Rich Fibrin +
ASA	American Society of Anesthesiologist
BML	Bone Marginal Loss
CBCT	Cone Beam Computed Tomography
FGFs	Fibroblastic Growth Factors
GBR	Guided Bone Regeneration
HI	Healing Index
IGFs	Insulin-Like Growth Factors
IL	Interleukins
I-PRF	Injectable Platelet-Rich Fibrin
KM	Keratinized Mucosa
L-PRF	Leukocyte and Platelet-Rich Fibrin
L-PRP	Leukocyte and Platelet-Rich Plasma
PA	Periapical Radiographs
PC	Platelet Concentrates
PDGF	Platelet-Derived Growth Factor
PPP	Platelet-Poor Plasma
P-PRF	Pure Platelet-Rich Fibrin
P-PRP	Pure Platelet-Rich Plasma
PRGF	Plasma Rich in Growth Factors
PRP	Platelet Rich Plasma
RBC	Red Blood Cells
ROI	Region of Interest
TGF-β	Transforming Growth Factor-Beta
TNF-α	Tumor Necrosis Factor-alpha
TSP-1	Thrombospondin 1
VAS	Visual Analogue Scale
VEGF	Vascular Endothelial Growth Factor

Abstract

Introduction: Tooth extractions are one of the most common procedures in dentistry, leading to important changes in the edentulous ridge. These may not allow a satisfactory positioning of dental implants and they may compromise the result of the prosthetic rehabilitation.

Immediate implants can be a viable treatment option, reducing the number of surgical procedures and the treatment period. However, one potential disadvantage is the gap formed between implant and buccal bone. To regenerate this bone defect, techniques have been developed that include the placement of different grafting materials such as xenografts and most recently, Leukocytes and Platelet-rich Fibrin (L-PRF).

The aim of this prospective split-mouth study is to analyze the effectiveness of L-PRF regeneration in post-extraction sockets with immediate implant placement, compared to regeneration with xenograft biomaterial (Bio Oss®).

Materials and Methods: Participants who fill the inclusion criteria will be part of both groups. Control and test site will be randomly assigned, at surgery day. At the surgery day, L-PRF membrane will be prepared and the implant will be placed in post-extraction socket. The gap will be filled with L-PRF membrane in test site and xenograft in control site. Clinical evaluation includes intra-oral photographs, healing tissue evaluation and keratinized soft tissue gain. Radiographic examination will consist in periapical radiographs and CBCT to measure bone marginal loss and bony defect regeneration. Follow-up appointments will be performed at day 10 and 30, 3 months, 6 months and 1 year after surgery. Participants will fill a questionnaire to self-assess post-operative pain. Finally, 1 year after surgery, implant's survival and success will be assessed.

Main Considerations: The literature indicates good results concerning L-PRF application. However, there is a limited number of studies and a lack of standardization, thus exposing the need for further RCTs assessing the effect of L-PRF on bone and soft tissue regeneration.

Key-words: L-PRF, soft-tissue, hard-tissue, regeneration, immediate implants

Resumo

Introdução: As extrações dentárias são um dos procedimentos mais comuns na área da medicina dentária.

Após uma extração dentária, o processo de cicatrização é iniciado com hemorragia e formação do coágulo, que é substituído por tecido de granulação. Seguidamente, é formada uma matriz de tecido conjuntivo provisória. Ao fim do primeiro mês pós-extração, verifica-se o preenchimento do alvéolo com osso imaturo que será progressivamente substituído por osso lamelar e medular. Consequentemente, este processo levará a alterações dimensionais no alvéolo.

Embora se verifique alterações dimensionais até ao primeiro ano após extração, são durante os primeiros 3 meses que a perda óssea e tecidular é mais acentuada. Esta perda é influenciada por diversos fatores como as variações individuais, o tamanho do alvéolo pós-extracional e a extensão do trauma provocado durante a extração. Um alvéolo localizado na mandíbula terá uma reabsorção maior do que na maxila assim como a tábua vestibular apresentará maior reabsorção do que a palatina/lingual.

A reabsorção do osso alveolar poderá comprometer a colocação de implantes, influenciando tanto o resultado estético como funcional. De forma a tentar prevenir situações de compromisso e diminuir o tempo de tratamento, os implantes imediatos surgiram como opções viáveis e alternativas à colocação de implantes diferidos.

A decisão de colocação de implantes imediata ou diferida irá depender de inúmeros fatores relacionados com a tábua óssea vestibular e a presença de tecidos moles pós-extração. De acordo com a classificação de Elian *et al.*, em situações onde os tecidos moles e duros estejam a níveis normais em relação à junção amelo-cementária do dente e mantêm-se com os mesmos níveis pós-extração, esse alvéolo é classificado como tipo I. Caso o nível ósseo esteja abaixo das referências anteriores, mas os tecidos moles encontrem-se a um nível normal, é classificado como tipo II. O alvéolo tipo III é o mais complexo de reabilitar, com reabsorção óssea e tecidular. Alvéolos do tipo I, são os mais fáceis de tratar e têm os resultados mais previsíveis.

Uma potencial desvantagem associada à colocação de implantes imediata é o espaço existente, após colocação do implante, entre a superfície do implante e a tábua óssea vestibular, uma vez que as dimensões do implante são inferiores às do alvéolo.

Na tentativa de preencher o espaço existente entre o implante e a tábua óssea vestibular e preservar o osso vestibular pré-existente, técnicas de regeneração têm sido desenvolvidas. Contudo, a literatura não é clara relativamente ao material mais eficaz para as técnicas de regeneração alveolar. Estas técnicas incluem a colocação de diferentes materiais de enxerto de osso autólogo, xenoenxerto, aloenxerto, regeneração óssea guiada e utilização de materiais bioativos, como a fibrina rica em leucócitos e plaquetas (L-PRF).

Um dos materiais mais estudados, o xenoenxerto, apresenta resultados benéficos com a sua utilização. Contudo, este material tem uma taxa de reabsorção lenta, levando à presença de partículas residuais que poderão interferir com a normal cicatrização alveolar bem como influenciar a qualidade do osso regenerado.

L-PRF é uma biomembrana constituída por fibrina rica em leucócitos e plaquetas derivadas de sangue autólogo e que não requer adição de anticoagulantes ou agentes gelificantes. É um biomaterial prático e fácil de preparar. Tem uma consistência sólida à base de fibrina, com uma distribuição tridimensional específica de leucócitos e agregados plaquetários. Graças à sua arquitetura, é mais denso que um PRP ou outros materiais de fibrina, influenciando a sua cinética biológica. Tem uma elevada resistência, flexibilidade e elasticidade aferindo-lhe capacidade de selar firmemente os tecidos biológicos. Caracteriza-se por polimerizar naturalmente durante a centrifugação. Quando formada, os seus constituintes irão libertar lentamente quantidades significativas de proteínas da matriz, citoquinas com características inflamatórias e cicatriciais e fatores de crescimento, promovendo a cicatrização tecidular e regeneração óssea. Uma vez que contém leucócitos, é capaz de estimular mecanismos imunes locais de defesa do organismo.

Posto isto, a membrana de L-PRF parece ter todas as propriedades necessárias para minimizar as mudanças que ocorrem em alvéolos pós-extracionais, tanto a nível ósseo como dos tecidos moles.

De acordo com a literatura existente, é expectável que um alvéolo preenchido com L-PRF conduza a uma menor reabsorção da tábua óssea vestibular, melhor cicatrização dos tecidos moles e uma menor dor pós-operatória.

O objetivo deste estudo prospetivo, do tipo *split-mouth,* é analisar o efeito de L-PRF na regeneração de alvéolos pós-extracionais com colocação imediata de implantes, comparado com regeneração com xenoenxerto (Bio Oss®).

Materiais e Métodos: Este estudo é um estudo cego prospetivo controlado e aleatorizado do tipo *split-mouth.* Sendo um estudo do tipo *split-mouth,* o mesmo paciente estará incluído no grupo teste e controlo. Deste modo, a seleção dos locais teste e controlo serão efetuadas de forma aleatória.

O grupo teste será definido como grupo de colocação de implante imediata e preenchimento do espaço com L-PRF enquanto que no grupo controlo, também de colocação de implante imediata, o espaço será preenchido com Bio Oss®.

Os critérios de inclusão incluem pacientes com indicação para extração e colocação de implantes bilaterais, saudáveis, com alvéolo tipo I e sem fratura da tábua óssea vestibular. Pacientes fumadores (>5cigarros/dia), ASA tipo III ou IV, alvéolo do tipo II ou III e lesão infeciosa difusa num dente adjacente ao que será extraído, são pacientes que preenchem os critérios de exclusão.

A membrana de L-PRF é preparada após colheita de sangue no dia da cirurgia e antes da mesma iniciar. É preparada segundo o protocolo descrito na literatura, utilizando o sistema Intra-Spin® da Intralock®. Após centrifugação, os constituintes sanguíneos encontram-se divididos por 3 camadas: a de eritrócitos, encontrados na porção inferior; a de plasma acelular (PPP, Plasma Pobre em Plaquetas) que se encontra no sobrenadante e uma camada designada "L-PRF clot" na qual as plaquetas estão concentradas. O “L-PRF clot” é recolhido e colocado num kit de preparação das membranas (Xpression^{TM}, Intralock®).

Após preparação das membranas, segue-se a cirurgia de extração dentária e colocação imediata de implante com regeneração. O paciente será submetido a anestesia local e o dente será extraído da forma mais atraumática possível, sem abertura de retalho ou osteotomia. O implante será colocado e o grupo teste terá o espaço entre o implante e a tábua óssea vestibular preenchido com L-PRF e o controlo com xenoenxerto (Bio Oss®). Ambos locais cirúrgicos serão suturados. Os Pacientes receberão informações sobre os cuidados pós-operatórios e prescrição terapêutica.

A avaliação clínica iniciar-se-á previamente à extração, com fotografias intraorais e avaliação da gengiva queratinizada, para posterior comparação com resultados pós-

extracionais. As consultas de controlo serão realizadas aos dias 10 e 30, 3 e 6 meses e 1ano após extrações. Nestas consultas serão realizadas fotografias intraorais, avaliação da cicatrização tecidular através do *Healing Index modified*,(até aos 3 meses) e avaliação da mucosa queratinizada através de sobreposição de imagens 3D obtidas pelo 3Shape TRIOS® e medição das diferenças (apenas aos 3 e 6 meses).

A avaliação radiológica será feita através de radiografias periapicais, para avaliar a perda óssea marginal, aos 0, 6 e 12 meses, e CBCT, para avaliar defeito ósseo, através de medições lineares aos 0 e 6 meses. Estas medições serão realizadas por um operador calibrado e independente ao estudo.

Os pacientes avaliar-se-ão quanto à sua dor pós-operatória, sentida nos primeiros 10 dias após extração, através do preenchimento de um questionário.

Finalmente, ao fim de 1 ano, serão avaliados a sobrevivência e o sucesso dos implantes, com os critérios pré-estabelecidos na literatura.

Considerações finais: Vários são os estudos existentes que reportam a eficácia da membrana de L-PRF nas mais variadas áreas da medicina dentária e, nomeadamente, na cirurgia oral.

Apesar da literatura existente indicar bons resultados com a utilização de L-PRF, revisões sistemáticas revelaram que existem um número limitado de estudos e falta de standardização em relação ao protocolo de utilização das membranas, com amostras e *follow-ups* curtos, expondo assim a necessidade de mais estudos randomizados e controlados para avaliar o efeito da membrana de L-PRF na cicatrização óssea e tecidular.

Este protocolo surge, portanto, como uma tentativa de adicionar à literatura resultados positivos, comparáveis com utilização de xenoenxerto, do efeito membrana de L-PRF para regeneração óssea e tecidular na colocação de implantes imediatos.

Palavras-Chave: L-PRF, tecidos moles, tecidos duros, regeneração, implantes imediatos

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1. Introduction

Tooth extractions are one of the most common procedures in dentistry, due to multiple causes such as decay, advanced periodontal disease, trauma, and others (Horváth, Mardas, Mezzomo, Needleman, & Donos, 2013). In addition to the function to maintain the masticatory system efficient or the facial esthetic, teeth play an important role in the maintenance of alveolar process dimensions and periodontal tissue support (Pietrokovski, Starinsky, Arensburg, & Kaffe, 2007). Following this, when a tooth is extracted, important changes will occur in the edentulous ridge (Araújo & Lindhe, 2009).

Nowadays, the importance of the prosthetic rehabilitation in edentulous spaces is recognized not only by clinicians but also by patients. The increasing demand of esthetics and comfort lead patients to desire fixed prosthetics solutions over removable ones, most of the times (Grunder, Gracis, & Capelli, 2005).

Clinicians often perform extractions without any planning of preservation of the alveolar ridge for late implant rehabilitation or without the evaluation of the possibility of immediate implant placement. This may lead to consequences for the remaining bone, making the entire rehabilitation process more difficult (Atieh et al., 2015).

1.1 Healing Process

Tooth extraction involves a mechanical trauma in soft tissues, periodontal ligament, bundle bone and the bone of the alveolar process (Araújo & Lindhe, 2009; Caneva et al., 2013). This leads to an inflammatory response that includes both hematopoietic and mesenchymal cells in the site (Amler, 1969; Cardaropoli, Araujo, & Lindhe, 2003). This way, the socket healing process may be divided in three phases: inflammatory, proliferative and modeling/remodeling (Araújo, Silva, Misawa, & Sukekava, 2015).

The healing process initiates immediately after tooth extraction, with a hemorrhage and blood clot establishment. Several inflammatory cells migrate to the wound in the first 2-3 days mediated by signaling molecules (i.e. growth factors and cytokines), like platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), fibroblastic growth factors (FGFs) and transforming growth factor-beta (TGF-β),

which are also responsible for cell differentiation and proliferation(Araújo et al., 2015; Lalani, Wong, Brey, Mikos, & Duke, 2003) . The combination of inflammatory and vascular cells with immature fibroblasts form the granulation tissue. Then, an additional establishment of a provisional connective tissue matrix occurs and it replaces the granulation tissue. This matrix is rich in collagen fibers and cells (Araújo et al., 2015; Barallat et al., 2014).

The proliferative phase initiates with the incorporation of vessels and bone forming cells within provisional matrix where projections of woven bone are arranged around the blood vessels. These projections will completely surround a vessel in order to form the primary osteon. The woven bone can be found in the healing socket 2 weeks after tooth extraction. In the first month, the socket is filled with woven bone (Araújo & Lindhe, 2005; Araújo, Sukekava, Wennström, & Lindhe, 2005).

Bone modeling and remodeling is the last phase of the socket-healing process. The woven bone will be progressively replaced by lamellar bone and bone marrow (Araújo & Lindhe, 2009; Barallat et al., 2014; Cardaropoli et al., 2003). At the same time, bone resorption takes place on the socket walls leading to dimensional changes of the alveolar ridge. The complete remodeling of the woven bone into lamellar and bone marrow may take several months or years and it depends on individual variability (Barallat et al., 2014; Lindhe et al., 2012).

The vertical resorption in the alveolar ridge is equal in both buccal and lingual sites but, since the lingual bone is usually wider than the buccal bone wall, the resorption at the buccal plate has greater magnitude compared to lingual wall. Bone resorption is less in mesial/distal sites than in buccal/lingual sites and it may be due to the presence of adjacent teeth. In total, the mean value of vertical bone loss is 1.24mm (± 0.11) at 6 months (Araújo & Lindhe, 2005; Discepoli et al., 2013; Tan, Wong, Wong, & Lang, 2012a).

Regarding horizontal bone resorption, the mean value is 3.79mm (± 0.23) at the level of the alveolar crest in the first 6 months, with more pronounced tissue loss in buccal aspect (Araújo & Lindhe, 2005; Tan et al., 2012a).

The molar teeth site have a greater value of reabsorption, but the resorption is more critical in the anterior region due to aesthetics demands (Pietrokovski et al., 2007).

Comparing differences between jaws, mandible resorbs more than maxilla (Araújo & Lindhe, 2005; Barallat et al., 2014; Smukler, Landi, & Setayesh, 1999).

Although dimensional changes can be observed up to 1 year after tooth extraction, it's during the first 3 months that the most statistically significant bone loss occurs, reaching values of 50% (Cardaropoli et al., 2003; Horváth et al., 2013; Schropp L, Wenzel A, Kostopoulos L, 2003; Vignoletti et al., 2012).

The end of socket-healing process is clinically observed by the closure of the socket with firm epithelized soft tissue and radiographic by bone fill of the socket. This will be influenced by biologic individuals' variations, alveolar socket size and the extended of socket trauma during the extraction (Araújo et al., 2015).

The alveolar bone resorption may not allow a satisfactory positioning of dental implants (Atieh et al., 2015; John, De Poi, & Blanchard, 2007) or compromise the aesthetic result of the prosthetic restorations (Nevins et al., 2006; Tan, Wong, Wong, & Lang, 2012b; Ten Heggeler, Slot, & Van Der Weijden, 2011; Vignoletti et al., 2012).

1.2 Implant Placement

In an attempt to rehabilitate the edentulous site, immediate implants can be a viable option, reducing the number of surgical procedures and the period of the treatment (Esposito, Koukoulopoulou, Coulthard, & Worthington, 2006; Evans & Chen, 2008; Lazzara, 1989).

The healing of bone tissues around implants is based mainly on correct osseointegration (Brånemark et al., 1969). During implant placement, the blood covers implant surface, coagulates on the surface creating a thick fibrin-platelet layer (Di Iorio et al., 2005). Blood constitutes the first matrix of healing during osseointegration (Alain Simonpieri, Del Corso, Sammartino, & Dohan Ehrenfest, 2009). Bone healing around implants follows the sequence of intramembranous osteogenesis starting with woven bone formation and followed later by formation of parallel-fibered bone and by lamellar bone. Bone remodeling also involves the bone–implant interface. Osseointegration is a dynamic process during which primary stability is substituted by secondary stability (Bosshardt, Chappuis, & Buser, 2017). Implant stability is referred as the absence of movement after implant placement (primary stability) or during osseointegration process (secundary stability) (Javed, Ahmed, Crespi, & Romanos, 2013).

The decision to choose an immediate over delayed implant placement will be influenced by the buccal bone plate and facial soft tissue. According to Ellian N. et al. (2007), when the facial soft tissue and buccal bone plate are at normal levels in relation to the cement-enamel junction of the pre-extracted tooth and remain intact post-extraction, sockets are classified as Type I. These types of sockets are the easiest and most predictable to treat. Type II or III sockets are the most difficult to treat. They are characterized by partial loss of buccal bone plate and maintenance of the facial soft tissues in type II or both bone and soft tissue loss in the buccal side in type III (Niles & Humpal-winter, 2007). Other factors that may influence the decision on immediate implant placement are related with absence of periapical pathology, presence of keratinized tissue, thick tissue biotype (AlKudmani, AL Jasser, & Andreana, 2017).

Cosyn et al. (2012), in a systematic review, concluded that patients with intact buccal bone, tick tissue biotype, flapless implant surgery with an immediate implant restauration might have a reduced risk of advanced midfacial recession (<10%) (Cosyn, Hooghe, & De Bruyn, 2012).

Although it was thought that immediate implant placement was associated with lower bone resorption, several studies showed failure on preventing bone modeling and maintenance of the socket wall (Araújo et al., 2015; Wang & Lang, 2012). Therefore, one of the potential disadvantages of immediate implants is the space created between the implant surface and the socket wall. Such gap is due to the difference in implant size and shape when compared to the extraction socket (AlKudmani et al., 2017; Wang & Lang, 2012). On the one hand, this gap will be filled with a blood clot and will create woven bone that will join the implant surface and the new bone formed during socket healing (Neiva et al., 2016a; Paolantonio et al., 2001). On the other hand, soft tissue down-growth may occur between the implant and socket wall (Esposito et al., 2006). This complication may compromise the esthetic result mainly in the anterior region and in patients with a high smile line (Clementini et al., 2015).

Buser et al. (1990) defined implant success by (Buser, Weber, & Lang, 1990):

- ➔ Absence of pain, foreign body sensation, dysesthesia;
- ➔ Absence of peri-implant infection with suppuration;
- ➔ Absence of mobility;
- ➔ Absence of persistent peri-implant radiolucency;

➔ Possibility for restoration;

However, new parameters have been introduced to evaluate success. These include health status and natural-looking peri-implant soft tissues, prosthodontic parameters, esthetics, and patient satisfaction (Gallucci et al., 2014).

Surgical techniques, including the use of bone grafting materials, were proposed to fill the space around implants in order to maintain the hard and soft tissues dimensions and architecture and regenerate lost bone where bony defects occurred (Gher, Quintero, Assad, Monaco, & Richardson, 1994).

Chen et al., in a randomized controlled trial, showed that a decrease of resorption in 15 to 20% of the horizontal resorption may occur when a bone graft with/without barrier membrane was placed simultaneously to immediate implant placement (Chen, Darby, Adams, & Reynolds, 2005).

1.3 Keratinized Mucosa

The width of keratinized mucosa (KM) around natural teeth is defined as the distance between the mucogingival junction and the free gingival margin (Lin, Chan, & Wang, 2013).

A minimum of 2mm of KM around natural teeth is required to maintain gingival health (Lang & Löe, 1972). An insufficient wide zone of KM shows more plaque accumulation (A. Temmerman, Cleeren, Castro, Teughels, & Quirynen, 2018). A higher plaque accumulation and gingival inflammation are demonstrated when a < 2mm of KM is observed around implants (Brito, Tenenbaum, Wong, Schmitt, & Nogueira-Filho, 2014).

An adequate KM might simplify restoratives procedures, allow a satisfactory oral hygiene without discomfort and improve esthetics (Souza, Tormena, Matarazzo, & Araújo, 2016).

1.4 Socket preservation/regeneration techniques

In an attempt to fill the gap and minimize the ridge resorption, several techniques have been developed to try to preserve post-extraction sockets. Even though the

occurrence of bone loss in both buccal and lingual side is always expected, the placement of graft materials has been as an ideal procedure to reduce the level of bone resorption (Iasella et al., 2003; Serino, Biancu, Iezzi, & Piattelli, 2003).

The preservation techniques include the placement of different grafting materials such as bone autografts (from the same patient), xenografts (from another species), allografts (from the same species), alloplastic materials (synthetic materials), and more recently, bioactive materials. These materials can be combined with resorbable or non-resorbable membrane (guided bone regeneration (GBR)) (Atieh et al., 2015; Vittorini Orgeas, Clementini, De Risi, & de Sanctis, 2013; Willenbacher, Al-Nawas, Berres, K?mmerer, & Schiegnitz, 2015). Some studies have shown that, with these techniques, the amount of ridge reduction in height ranged from +1.3mm to -2.64mm and in width varied from -1.2mm to 2.64mm, depending on the type of materials used (Chan, Lin, Fu, & Wang, 2013; Ten Heggeler et al., 2011).

Willenbacher et al., on a meta-analysis, concluded that about 0.95mm up to 1.12mm of mean apico-coronal ridge height and 1.31mm to 1.54mm of mean bucco-oral ridge width can be preserve using alveolar ridge preservation techniques compared to natural healing with a defined 6 months of follow up (Willenbacher et al., 2015).

Different kinds of graft materials may influence the socket healing and may compensate for the buccal loss (Araújo et al., 2015; Chan et al., 2013). However, the presence of residual particles is a concern since they might interfere with normal healing (Avila-Ortiz, Elangovan, Kramer, Blanchette, & Dawson, 2014; Becker, Becker, & Caffesse, 1994). Another fact that may be relevant is the bone quality, which is dependent on the resorption rate of the grafting material used, as well as the ability to promote bone formation (Chan et al., 2013).

Bone quality might affect implant success in two aspects: the degree of bone-to-implant contact and the achievement of primary stability. If the residual grafts do not integrate well with bone, it is expected a decrease of bone density (Chan et al., 2013). Consequently, implants must be placed more apically to previous socket to achieve primary stability (Tomasi et al., 2010).

The greater percentages of residual graft particles were seen in sockets treated with xenografts and allografts (Barallat et al., 2014). Besides that, systematic reviews revealed that the use of a xenograft or an allograft had a beneficial effect in bone

preservation compared to alloplastic materials (Atieh et al., 2015; Avila-Ortiz et al., 2014). Xenograft is one of the most studied materials (Atieh et al., 2015).

Araújo et al. (2011), in an experimental study in dogs, showed that applying Bio-Oss Collagen® (xenograft) in the gap during immediate implant placement, modified the process of hard tissue healing, provided additional amounts of hard tissue, improved the level of marginal bone-to-implant contact and prevented soft tissue recession (Araújo, Linder, & Lindhe, 2011).

The literature isn't clear about which material is the most effective for preservation and regeneration techniques (Atieh et al., 2015). GBR procedures and bioactive materials, such as Leukocyte and Platelet-rich fibrin (L-PRF), show promising results (Vignoletti et al., 2012).

1.5 Leukocyte and Platelet-Rich Fibrin

The L-PRF is a bioactive material, classified as a second generation of platelet concentrates (PC) which appeared to improve and simplify the use of these preparations (Dohan et al., 2006b).

1.5.1 Platelet Concentrates

The PC was developed to reinforce the natural wound healing (Agrawal, 2017). It was first used, in the early 70s, as a fibrin glue to improve wound healing (Davis et al., 2014a). Then, the first generation of PC was introduced. These concentrates include platelet rich plasma (PRP) and plasma rich in growth factors (PRGF) both of which require anticoagulants to avoid coagulation (Anitua, 2001; Marx, 2001). The second generation, L-PRF, was developed in 2001 by Dr. Joseph Choukroun, specifically in oral and maxillofacial surgery. The L-PRF is a three-dimensional healing matrix with leucocytes and platelets rich-fibrin derived from autologous blood (Dohan et al., 2006b, 2006c; Dohan Ehrenfest, Rasmusson, & Albrektsson, 2009).

The classic PRP technique can be performed with manual kits or automated instruments, using 2 spins. There are many variations of the classic PRP with diverse speed centrifuge, amount of blood required, presence of leukocytes, type of instrument and platelet activation ways (Davis et al., 2014b). The PRGF have a lower platelet concentration, compared to others PRPs, nearly none leukocyte and it can be used like an

injectable solution or a fibrin gel, if it is activated (Dohan Ehrenfest et al., 2012; Weibrich, Kleis, Hitzler, & Hafner, 2005). The fibrin gel is fragile and unstable (Dohan Ehrenfest et al., 2012). The literature available on PCs is confusing and controversial due to the lack of appropriate characterization of the different products (Agrawal, 2017; Dohan Ehrenfest, Lemo, Jimbo, & Sammartino, 2010; Dohan Ehrenfest, Rasmusson, et al., 2009).

To clarify all available techniques related to PC's, a classification was designed depending on leukocyte content and fibrin architecture. Therefore, the PC's was regrouped in 4 families: Pure Platelet-Rich Plasma (P-PRP), Leukocyte and Platelet-Rich Plasma (L-PRP), Pure Platelet-Rich Fibrin (P-PRF) and L-PRF. The first two families are liquid suspensions, with or without leukocytes, respectively L-PRP or P-PRP. After activation with specific agents such thrombin, calcium chloride and others, these preparations become fibrin gels. The last two families are solid fibrin material also with or without leukocytes, respectively. The platelet activation occurs during centrifugation and it can be natural with L-PRF or artificial with P-PRF (Dohan Ehrenfest et al., 2012; Dohan Ehrenfest, Rasmusson, et al., 2009).

The procedures related to PRP's have multiple steps and can be complex, which could compromise and vary the quality of the resulting material (Davis et al., 2014a; Dohan Ehrenfest et al., 2012). Also, these preparations are expensive and difficult to use on a daily basis (Del Corso, Mazor, Rutkowski, & Ehrenfest, 2012; Andy Temmerman et al., 2016).

Unlike PRP's, the L-PRF does not use any kind of anticoagulant or gelling agent and polymerizes naturally through centrifugation (Dohan et al., 2006a).

1.5.2 Protocol for L-PRF preparation

The L-PRF protocol is simpler than other PC protocols and includes venipuncture for a 10ml tube which is immediately centrifuged at 2700rpm for 12 minutes (Dohan Ehrenfest, Del Corso, Diss, Mouhyi, & Charrier, 2010). The previous protocol was 3000rpm/10minutes (Dohan et al., 2006a) but this protocol results in less resistant and polymerized L-PRF (Agrawal, 2017). When blood contacts with the tube walls, the platelets initiate the coagulation cascade (Davis et al., 2014b). The circulating thrombin will transform the fibrinogen into fibrin (Dohan et al., 2006a). During centrifugation,

blood coagulation and blood elements separation are taking place at the same time (Shah, MG, Thomas, & MEHTA, 2017).

After the centrifugation, three layers are formed: red blood cells (RBC) at the base, the L-PRF clot in the middle and platelet-poor plasma (PPP) at the top (Dohan Ehrenfest, Del Corso, et al., 2010). The L-PRF clot can be used as a membrane if it's compressed or it can be used directly as a clot or mixed with bone material (Del Corso et al., 2012; Dohan Ehrenfest et al., 2018; Dohan Ehrenfest, Del Corso, et al., 2010). To compress the clot is necessary a specific metal box (Xpression kit™, Intralock®) that compresses gently by gravity or into a fibrin cylinder (Dohan Ehrenfest, Del Corso, et al., 2010). The membrane it's done after 5minutes. All exudate will remain in the bottom of the box and may be used to hydrate the membrane, if it is required (Dohan Ehrenfest, Del Corso, et al., 2010; Miron et al., 2017; Toffler et al., 2009) (figure1).

It's recommended to draw the blood before the surgery, since the surgery itself may cause platelet activation which can interfere with the procedure (Agrawal, 2017; Man, Plosker, & Winland-Brown, 2001).

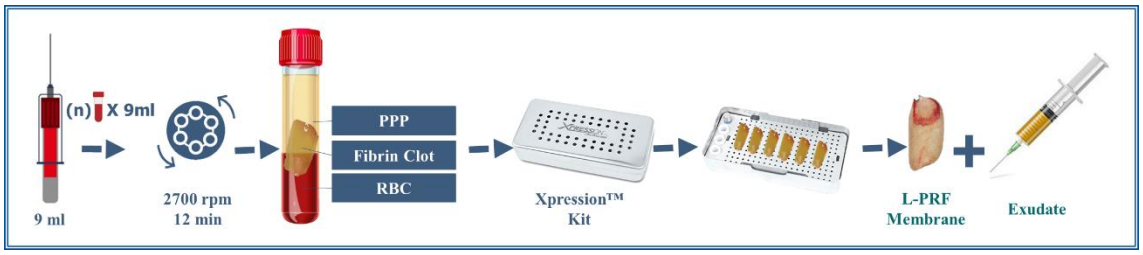

Figure 1: L-PRF membrane protocol

1.5.3 Characterization of L-PRF

The L-PRF is characterized as an optimized blood clot (Dohan Ehrenfest, Del Corso, et al., 2010). The natural clot is composed by approximately 95% erythrocytes, 5% of platelets and less 1% of leukocytes (Agrawal, 2017; Davis et al., 2014b; Mehta & Watson, 2008). In contrast, L-PRF contains almost all the platelets (±95% of initial blood) and more than 50% of the leukocytes of the withdraw blood. In that way, L-PRF has a strong fibrin structure with a specific three-dimensional distribution of platelet aggregates and leukocytes (Dohan Ehrenfest, Del Corso, et al., 2010), making it denser than other fibrin-rich materials and influencing its biologic kinetics (Cieslik-Bielecka, Choukroun, Guillaume, & Dohan Ehrenfest, 2012). This biomaterial has a high resistance, flexibility,

elasticity and it's safe, given its capacity to seal biological tissues (Davis et al., 2014b; Dohan et al., 2006a).

To promote tissue healing and bone regeneration, the L-PRF membrane slowly releases a significant amount of matrix proteins such fibronectin, vitronectin and Thrombospondin 1 (TSP-1), inflammatory and regenerative cytokines (interleukins IL-1β, IL-6 , IL-4 and Tumor Necrosis Factor-α (TNF-α)) and growth factors like TGF-β, PDGF, Vascular Endothelial Growth Factor (VEGF), IGF-1 and FGF during, at least, the first 7 days and, due this, it can be consider a living tissue graft (Cieslik-Bielecka et al., 2012; Davis et al., 2014a; Dohan et al., 2006c; Dohan Ehrenfest, de Peppo, Doglioli, & Sammartino, 2009). Growth factors together with cytokines can promote the differentiation of osteoblast as well as inhibiting the function of osteoclast (Jang et al., 2010).

Dohan et al (2006) quantified the presence of IL-1β, IL-6, TNF-α (inflammatory cytokines) and IL-4, VEGF (healing cytokines) in the PRF clot. The results showed that clot exudates have a higher concentration of IL-1β, IL-6, TNF-α and IL-4 compared to PPP. Only VEGF present a higher concentration in PPP. Thus, the PRF clot could be considered as an immune organizing node with the ability to show a feedback control of the site inflammation (Dohan et al., 2006c; Giannini et al., 2015).

It is the structure of fibrin that is responsible for the slow release of growth factors and matrix proteins (Davis et al., 2014a; Werner & Grose, 2003). Furthermore, the fibrin binding with numerous different growth factors may explain the fibrin angiogenesis induction (Choukroun et al., 2006b).

Platelets are the main cell responsible for biologic activity of PRF (Shah 2017). These cells are responsible for the first phase of blood clotting which includes adhesion, activation and aggregation of the platelets. Some studies showed that platelets from L-PRF releases growth factors biologically active involving tissue repair mechanisms, like chemotaxis, cell proliferation, angiogenesis, extracellular matrix deposition and remodeling (Singh, Kohli, & Gupta, 2012).

L-PRF also revealed a good capacity of proliferation and differentiation of osteoblasts and gingival fibroblasts (Marrelli & Tatullo, 2013).

Moreover, L-PRF contains leukocytes that induces local defense immune mechanisms (Davis et al., 2014a). Neutrophils are the first type of leukocytes to migrate to the wound to clean and prevent infection in early inflammatory phase of healing (Davis

et al., 2014b). After this, monocytes are recruited and differentiate to macrophages. This type of cell, phagocytes dead neutrophils and debride the wound – killer macrophage. Macrophage also release growth factors and cytokines, recruit cells to the wound and stimuli angiogenesis, helping the progress from initial inflammatory phase to proliferative phase – repair macrophage. In addition, the white cells may can increase the platelet production by megakaryocytes. These cells, in turn, will recruit more leukocytes that will accelerate the healing process and prevent infection (Davis et al., 2014b; Dohan et al., 2006c; Dohan Ehrenfest, Del Corso, et al., 2010). Leukocytes also express many proteinases limiting the inflammatory response in wound site and adjacent tissues and remodeling the extracellular matrix in normal wound healing (Davis et al., 2014b; Lingen, 2001) which make leukocytes a key factor in wound healing (Davis et al., 2014b; Martin & Leibovich, 2005).

Platelets and leukocyte distribution is not uniform. Higher concentrations of these are found in an immediate layer between RBCs and fibrin clot. Therefore, it's necessary to collect a small RBC layer to ensure that the most leukocytes and platelets as possible are in the membrane (Dohan Ehrenfest, Lemo, et al., 2010).

L-PRF and natural blood clot main difference is the fact that L-PRF is prepared outside the surgical site making it very stable and homogeneous. This biomaterial is easy to prepare and to place in the surgical site (A Simonpieri et al., 2012). Nevertheless, time is decisive factor to obtain a successful membrane (Davis et al., 2014a; Dohan Ehrenfest, Lemo, et al., 2010; Madurantakam, Yoganarasimha, & Hasan, 2015). If the time between to collect the blood and to centrifugate extend overly long, the fibrin will polymerize in a diffuse way in the tube and a small blood clot without consistency will be obtained (Davis et al., 2014b; Dohan et al., 2006b; Madurantakam et al., 2015).

Another aspect that has influence on L-PRF quality is the centrifuge device. Table centrifuges available on the market have higher vibration intensity compared to the original L-PRF centrifuge (Intra-spin®) (Dohan Ehrenfest et al., 2017). When centrifuges with a higher vibration level are used, the L-PRF clot obtained can be entirely distorted (Dohan Ehrenfest et al., 2017).

By putting all of this under consideration, L-PRF seems to gather all that is required to minimize the dimensional changes that occur in post extraction socket and improve the socket healing, besides the fact that act as a bio-barrier which can protect the

implant from the oral environment (Dohan Ehrenfest et al., 2012). According to previous data, it's expectable that socket with L-PRF lead to less resorption in buccal bone, better soft tissue healing and less post-surgical pain (Hauser et al., 2013; Kumar et al., 2015; Singh et al., 2012; Suttapreyasri & Leepong, 2013).

1.5.4 Variations of L-PRF technique

Several variations of the conventional protocol, described by Choukroun et al, have been introduced (Shah et al., 2017). These different protocols include the obtained advanced platelet-rich fibrin (A-PRF), injectable platelet-rich fibrin (i-PRF) and advanced platelet-rich fibrin + (A-PRF^{+}) (Ghanaati et al., 2014; Mourão, Valiense, Melo, Mourão, & Maia, 2015; Shah et al., 2017).

A table below show the different preparations protocols (table1).

PRF types	**Year**	**Centrifugation time (min)**	**Centrifugation speed (rpm)**
L-PRF	2004 (Choukroun)	12	2700
A-PRF	2014 (Ghanaati)	14	1300
A-PRF^{+}	2016 (Fujioka Kobayashi, Miron)	8	1300
i-PRF	2015 (Mourão)	3	700

Table1: differences between different PRF protocols.

Regarding A-PRF, the authors defend that with a slower centrifugation combined with increased spin time, the number of viable cells are higher compared to L-PRF. This would be profitable if it means an increased release of growth factors and cytokines. Nevertheless, the literature is contradictory and limited about these kind of PRF and some studies demonstrate that this protocol produces shorter membranes, lighters and more squashed bodies (Shah et al., 2017).

In 2016, the A-PRF^{+} protocol was introduced with the same speed centrifugation, but with a decreased time of spin comparing to A-PRF. The concept was increasing the cell number in the PRF matrix. it was observed an increased release of PDGF, TGF- β1, EGF and IGF which might optimize growth factor production and cellular response to PRF (Fujioka-Kobayashi et al., 2017; Shah et al., 2017).

i-PRF was presented to address the limitations of L-PRF, that isn't possible to be injectable, and to replace PRP in its indications. The timing spin is shorter than other products in order to do not generate a gel consistency. It has been used for mixing with

bone grafts, which on completion of the coagulation process forms a gel consistency with the graft particles incorporated in the graft (Mourão et al., 2015; Shah et al., 2017).

All the alternative PRF aim to improve the growth factor which mediates the biologic effects and the cellular activity. Yet, further animal and clinical studies are needed to support the literature about these biomaterials (Shah et al., 2017).

1.5.5 Advantages of L-PRF

The current literature reports several advantages concerning L-PRF (Castro et al., 2017b; Choukroun et al., 2006a; Davis et al., 2014a; Dohan et al., 2006c):

- ➔ Easy to prepare and low-priced;
- ➔ No need to add any anticoagulant or gelling agent;
- ➔ It's made by autologous blood;
- ➔ It's living tissue, releasing growth factors during, at least, 7 days;

1.5.6. L-PRF applications in dentistry

Regarding the current applications of L-PRF in dentistry, this biomaterial is indicated to:

- ➔ Oral surgery - Sinus elevation procedures; Vertical and horizontal ridge augmentation; Alveolar ridge preservation; Guided bone regeneration procedures; Implant soft and hard tissue regeneration; Implant coating; Surgical site infections (alveolitis); Filling of cystic cavity;
- ➔ Endodontics - regenerative therapy; periapical lesions;
- ➔ Periodontics - Furcation and infra-bony defects; root coverage of gingival recessions; periimplantitis;

(Stipho & Talic, 2001)(Lollobrigida et al., 2018; Miron et al., 2017; Nanditha, Chandrasekaran, Muthusamy, & Muthu, 2017; Shah et al., 2017)

1.6 L-PRF in Immediate implant placement

The response of peri-implant hard and soft tissue filled with L-PRF following implant placement is not well documented (Boora, Rathee, & Bhoria, 2015).

The use of L-PRF may decrease the risks of infectious phenomena and reduce postoperative edema and pain, especially in the first days after the surgeries (Agrawal, 2017; Kumar et al., 2015; Miron et al., 2017; Alain Simonpieri et al., 2009).

Considering how L-PRF works, the literature suggests that L-PRF is able to reduce the bone resorption and improve bone healing after tooth loss, preserve the quality and density of the residual ridge (Castro et al., 2017b; Miron et al., 2017).

Moreover, beneficial effects in implant surgery are suggested when L-PRF is applied, speeding up the soft tissue wound healing with gap filling during immediate implant placement (Castro et al., 2017b; Miron et al., 2017). Previous studies compared implant placement with L-PRF showing that L-PRF decreases the marginal bone loss (Lee et al., 2012; Öncü & Erbeyoğlu, 2017) and stimulate gingival healing and bone healing surrounding implant (Dohan Ehrenfest et al., 2012; Öncü & Erbeyoğlu, 2017).

According to Lollobrigida et al. (2018), treatment of implant surfaces with liquid PRF leads to the formation of a stable and dense fibrin layer in direct contact with the implant surface improving the early stages of osseointegration (Lollobrigida et al., 2018).

L-PRF contributes to create a sufficient amount of keratinized tissue with a minimum invasive technique, low costs and low patient morbidity (A. Temmerman et al., 2018).

L-PRF may provide a continuous pathway for osteogenic cell migration, facilitate angiogenesis, chemotaxis and cell proliferation between the healing socket walls and the implant surface, reducing the osteointegration time (Coelho & Jimbo, 2014; Neiva et al., 2016b; Öncü, Bayram, Kantarcı, Gülsever, & Alaaddinoğlu, 2016).

2. AIM

The aim of this prospective split-mouth study is to analyze the effectiveness of L-PRF regeneration in post-extraction sockets with immediate implant placement, compared to regeneration with xenograft biomaterial (Bio Oss®).

3. Hypothesis

The null hypothesis (H0) of the present study is: "L-PRF membranes do not show any advantages in post-extraction socket healing with immediate implant placement".

It will be tested the hypothesis (H1) that L-PRF membranes have a positive influence in post-extraction sockets with immediate implant placement.

3.1 Outcomes variables

The primary outcome measures will be defined as a gain of keratinized tissue (mm), tissue healing and alveolar ridge bone resorption (mm) using L-PRF membrane versus Xenograft. Keratinized tissue gain (mm) will be assessed by digital overlapping of 3Shape TRIOS® Scan, at 3 and 6 months after tooth extraction. Tissue healing will be assessed by modified healing index and standardized photographs at day 10, 30 and 3 months after tooth extraction. Alveolar ridge resorption will be evaluated by marginal bone loss and bone defect. Marginal bone loss will be measured with periapical (PA) radiograph (mm) at 6 and 12 months after tooth extraction and bone defect on cone beam computed tomography (CBCT), considering linear measurements (mm), at 6 months.

The secondary outcomes will be implant survival and success and participant self-assessment. Implant survival will be defined as maintenance in mouth after 1 year and implant success will be evaluated according success criteria defined by Buser et al 1990, at 1 year after implant placement. Self-assessment will be a subjective evaluation using visual analogue scale (VAS). The participants will evaluate post-operative pain using a systematic questionnaire during the first 10days after tooth extraction.

4.Materials and Methods

4.1 Study design and Randomization

This study will be a prospective single blind randomized-control trial with split mouth design. It will be multicentered at the Faculty of Dentistry, University of Lisbon (FMDUL) and at a private practice (Institute of Implantology®).

Participants will be part of both two groups and sockets will be randomly assigned using a computer randomization system, at the day of surgery. Post-extraction sockets will be submitted to regeneration with L-PRF membranes in the test group, whereas control group's post-extraction sockets will be regenerated with xenograft material, Bio Oss®. Radiographic evaluation will be performed by an independent operator, blinded to the randomization process.

This study will be conducted in accordance to the Helsinki Declaration of 1975, revised in 2013. An informed consent was made (appendix 1) and approved by the Ethic Committee for Health from Faculty of Dentistry, University of Lisbon (Comissão de Ética para Sáude-FMDUL) (appendix 2).

4.2 Sample Size calculation

A total of 12 patients (24 samples) will enter in this two-treatment study. The probability is 90 percent that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments is 1.389 times the standard deviation.

4.3 Inclusion Criteria

The criteria for inclusion in this study will be as follows: patients from FMDUL or Institute of Implantology® with indication for bilateral paired teeth extraction and immediate implant placement in the maxilla or mandible, type I sockets, ASA (Physical Status Classification System, American Society of Anesthesiologist) I or II, patients without bisphosphonate treatment or systemic disease that may interfere with healing, aged 18 years or over, non-smokers or light smokers (<5/day).

4.4 Exclusion criteria

The exclusion criteria include: patients with no indication for immediate implant placement, type II or III sockets, sockets without fracture of the buccal bone wall, ASA III or IV, uncontrolled diabetes, use of immunosuppressant medication, heavy smokers, pregnant woman or patients with a diffuse infectious process next to the site to be intervened.

4.5 L-PRF preparation according to IntraSpin™ system

At the surgery day and before it begins, a venipuncture will be performed, with a 21G butterfly needle (BD Vacuntainer®, Safety-Lok™ Blood-collection Set with Pre-Attached Holder) on median basilica vein, median cubital vein or media cephalic vein.

Blood will be drawn into sterile plastic red cap tubes (BD Vacuntainer® CAT-clot activator tube) with 9ml capacity and without any anticoagulant, and then centrifuged at 2700 rpm for 12 minutes (IntraSpin, IntraLock®, Florida, USA) according to methods previously described in the literature (Öncü et al., 2016; Pinto, Teughels, Temmerman, & Quirynen, 2016).

After centrifugation, 3 layers are formed: PPP on the top, L-PRF fibrin clot in the middle and RBC at the bottom. The L-PRF fibrin clot is then separated from the red element, with surgical tweezers and scissors, in order to collect as many platelet and leukocytes as possible, as they are rather concentrated in an immediate layer located between RBCs and the fibrin clot (Davis et al., 2014a; Dohan Ehrenfest, Del Corso, et al., 2010).

The fibrin clot is transferred to a sterile kit (Xpression™ box) designed to compress L-PRF clots, through gravity, and it forms a 1mm thickness L-PRF membrane. The exudate released by these compressions will be later used (Dohan Ehrenfest, Del Corso, et al., 2010; Pinto et al., 2016).

After compression, The L-PRF membrane is ready to be used. The membrane has a red area which represents the “face” side, where leukocytes and platelets are present with a higher concentration (Dohan et al., 2006b).

The success of this technique entirely depends on the speed of blood collection and transfer to the centrifuge. Quick handling is the only way to obtain a clinically usable PRF clot - there must be a time range no longer than 1 min between blood collection and its transfer to the centrifuge (Crisci et al., 2017; Dohan et al., 2006b).

4.6 Surgical technique

At the beginning of the surgery, each patient will be submitted to local infiltrative anesthesia (Articaíne 4% with epinephrine 1: 200 000, Inibsa, Sintra, Portugal), both buccal and palatal or lingual. Following the anesthetic procedure, the surgeon will perform atraumatic tooth extraction, to preserve the integrity of the alveolar buccal plate, followed by alveolar curettage and irrigation with saline solution.

No incision, flap release or osteotomy will be performed during the surgery. However, odontossection will be required in cases of single-rooted teeth with ankylosis or multi-rooted teeth, to prevent buccal bone plate fracture.

In every case, an immediate implant (Straumann® SLA® bone level) will be placed in the former tooth position, with a minimum primary stability value of 45 Ncm. The operator will be free to choose implant lengths and diameters according to clinical indications.

The preparation of the surgical site is what will distinguish the groups participating in the study. Hence, according to randomization at surgery, each participant will be submitted to regeneration procedures with L-PRF membranes in the post-extraction sockets (test group) and will be submitted to regeneration procedures with xenograft (control group).

Control group – Xenograft:

The immediate implant will be placed in a palatal/lingual position into the extraction socket (Araujo et al 2006). Following implant placement, the residual gap will be filled with xenograft material - deproteinized bovine bone mineral (Small Geistlich Bio-Oss® granules (0.25 – 1 mm), Geistlich Pharma, Wolhusen, Switzerland). According to the manufacturer's instructions, Bio-Oss® will be mixed with saline solution before application.

Test group - socket treatment with L-PRF application:

The immediate implant will be placed in a palatal/lingual position into the extraction socket, 5 min after immersion in the exudate obtained from L-PRF clots, according to previous described methodology (Lollobrigida et al., 2018). The space between the titanium surface and the buccal bone wall will be filled with L-PRF membranes, according to the following protocol:

1. L-PRF membranes are introduced inside the socket, between the buccal bone wall and the implant. Membranes are placed one by one, and compressed firmly, until no more free spaces are left. Excess of serum is absorbed with a gauze.
2. The socket will be covered with, at least, a double layer of L-PRF membranes (Pinto et al., 2016).

Finally, a tension-free monofilament non-resorbable cross suture will be placed on the socket in order to maintain the membranes in place.

Patients, in both groups, will go through a strict therapeutic regimen with Amoxicilin 875 mg in association with Clavulanic acid 125 mg (starting the day before the surgery and taking at each 12hour, for 8 days), 600 mg of Ibuprofen (at each 12hour for 3 days) and 1g of Paracetamol (at each 8hour, for the first three days). Patients will also be advised to use a mouthrinse with chlorhexidine (0.12% Perio Aid®, Elgydium), twice a day, for a period of eight days.

4.7 Clinical evaluation

Clinical evaluation initiates before the extraction with intraoral photographs and keratinized soft tissue assessment to define the baseline references to later compare with post-extraction results.

Follow-up appointments will be performed at day 10 and 30, 3 months, 6 months and 1 year after surgery. In the first follow-up appointment, sutures will be removed and the following variables will be accessed at every follow-up appointment, by a calibrated operator:

4.7.1 Intra-oral photographs

Occlusal and lateral photographs (to compare the pre-surgical images and access the site healing evolution).

4.7.2 Healing tissue evaluation

The healing tissue will be evaluated by an adapted version of the Healing Index (HI) described originally by Masse et al. and developed to evaluate tissue that is healing with primary closure after periodontal surgery (Masse et al., 1993). This adapted HI was modified to evaluate the healing tissue without primary closure and comprehends 3 scoring levels for each of the 4 considered parameters (Mozzati, Gallesio, Di Romana, Bergamasco, & Pol, 2014):

- ➔ Tissue color:
  - 1 = 100% of gingiva pink;
  - 2 = <50% of gingiva red, hyperemic, movable;
  - 3 = >50% of gingiva red, hyperemic, movable;
- ➔ Color and consistency of the healing tissue:
  - 1 = close grained, pink;
  - 2 = soft, red;
  - 3 = fragile, greenish or grayish;
- ➔ Suppuration:
  - 1 = absent;
  - 2 = absent but pronounced amount of plaque around socket walls;
  - 3 = pronounced;
- ➔ Bleeding:
  - 1 = absent;
  - 2 = induced by palpation;
  - 3 = spontaneous;

Therefore, through the sum of the indicators' values, the scoring scale may range from 4, corresponding to excellent healing, to 12, indicating severely impaired healing.

4.7.3 Keratinized tissue assessment

Keratinized tissue will be assessed by digital overlapping of 3Shape TRIOS® Scan, at 3 and 6 months post-extraction. A Scan will be performed prior to tooth extraction, to define baseline reference.

The images obtained by 3Shape TRIOS® (STL extension file) will be superimposed on the computer software (Geomagic studio 9 and qualify 9, geomagic, Research Triangle Park, NC, USA) to analyze dimensional changes of the KM surrounding the implant, according to previous described methodology by Schnutenhaus et al. (2018) (Schnutenhaus, Martin, Dreyhaupt, Rudolph, & Luthardt, 2018). Thus, the KM differences, since baseline at 3 months and 6 months, will be measured. Reference structures will be defined for the exact superimposition of the images. Then, the KM surrounding the implant, Region of Interest (ROI), will be determined. The scan images will be reduced in concordance. The ROI will be defined as follow:

→ Mesiodistal - orientation up to the adjacent teeth;

→ Buccal - to a maximum height of the bottom of the vestibule;

→ Palatal/Lingual - in the same vertical height as buccal;

→ Extraction socket – at the maximum point of the gingival margin surrounding the socket;

3D measurement → The three-dimensional analysis method requires a separation into positive and negative measurement points. Only the negative values will be included in the analysis, while the positive values must be treated as artifacts.

4.8 Radiographic examination

Prior to each surgery, complementary exams are required for treatment and surgical planning. Hence, each patient will be submitted to a pre-surgical CBCT scan (CBCT Planmeca ProMax Dimax 3 Digital Plan/Ceph) with a 0.20 voxel size, 80 kV and 15 mA, within an exposure time of 12 seconds according to manufacturer instructions. Cross-sectional images will be reconstructed to a 0.6 mm slice thickness and high artefact removal will be applied. A second CBCT scan will be performed immediately after surgery (T0), not only to access implant position but also to provide a reference value for the bony defect regeneration.

Finally, after a minimum period of 6 months, a third CBCT scan will be taken (T1).

Additionally, PA radiographs will be taken, through the paralleling technique, to measure the peri-implant marginal bone-level changes comparing to day 0, 6 months and at 1year post-extraction.

An independent, calibrated operator will analyze, retrospectively, each PA radiograph and CBCT scan.

4.8.1 Radiographic measurements

Bone defect: To assess bone defect in both test and control group, CBCT analyses will be performed. The postoperative scans will be spatially matched to the preoperative CBCT based in the panoramic cut. It will be measured the distance from the nasopalatine canal (midline reference) to the center of each implant, allowing standardization of each location, per implant, from baseline, up to 1year follow-up (figure 2).

Subsequently, standardized linear measurements were made on cross-sectional images generated perpendicular to the occlusal plane using the same reference points and lines. The vertical reference line will be the implant midline. The horizontal reference line will be drawn at midpoint of the implant and will be perpendicular to the vertical line. The horizontal line will be measured from implant midpoint, intersected by vertical and horizontal line, to the most buccal portion of the bone plate (figure 3).

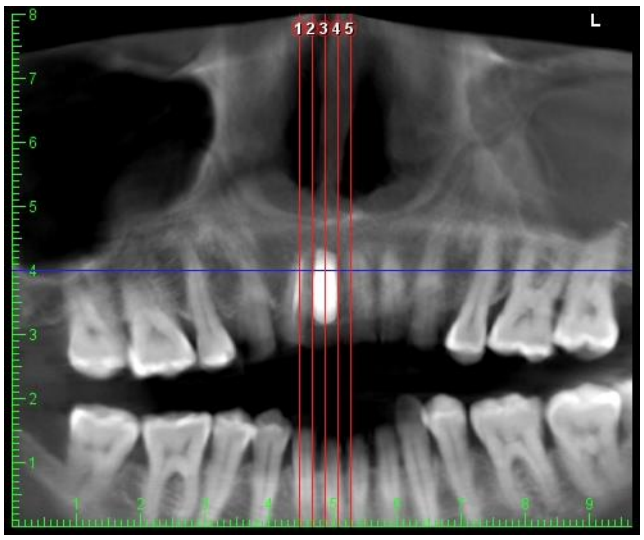

Figure 2 Representative image of a panoramic cut

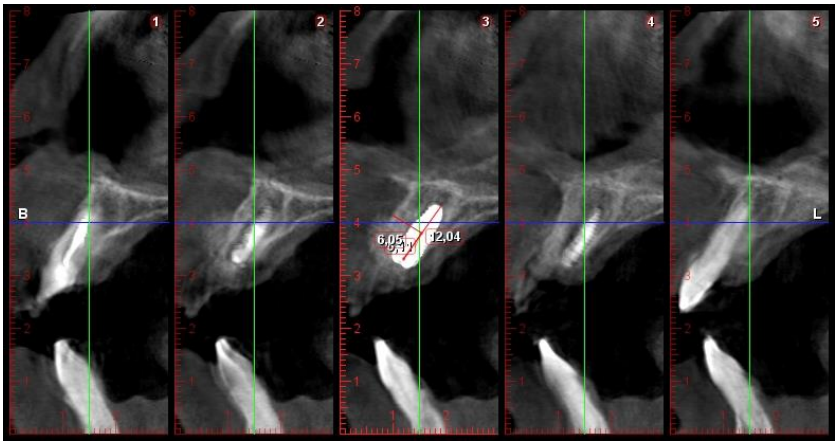

Figure 3 Representative image of sagittal cut with linear measurements

Bone Marginal Loss (BML): In order to evaluate BML, PA radiographs will be taken using a paralleling modified device: with a Rim XCP, a putty mold will be obtained from the patient bite in the area where the PA radiograph will be taken, so that the film is always placed in the same position. There will also be a mold made between the ampoule and Rim XCP positioner, so that the ampoule always takes the same declination and direction, which allows radiographs standardization.

The baseline, at the day of implant placement, will be compared with the 6months and 1year post-extraction. Measurements will be performed with a computer software (Kodak 900), according to Boora and co-workers (Boora et al., 2015). The reference line will be defined by the most coronal point of the abutment. The bone level will be established at the point of bone-to-implant contact. Measurements will be made in millimeters at the mesial and distal aspect of implants by placing perpendicular line from the static reference line to the bone level.

4.9 Self-Assessment

Participants will be given information and instruction to answer a questionnaire to self-assess post-operative pain. The participants will answer, daily, for both test and control site during the first 10 days following the surgery. To assess post-operative pain, the Brazilian version of the short-form McGill Pain Questionnaire will be used (Da C. Menezes Costa et al., 2011). The patient will be asked to fill in the VAS-scale. The VAS

has a score that ranges between 0 that equals “no pain” and 10 that equals “worst imaginable pain” (appendix 3).

Questionnaires will be collected in the first follow-up visit – at day 10.

4.10 Implant’s Survival and Success

4.10.1 Implant survival

Implant survival will be defined as maintenance in mouth at 1 year after implant placement.

4.10.2 Implant success

Implant success will be assessed at 1 year after implant placement, according to success criteria defined by Buser et al 1990, following the bellow parameters:

- ➔ Plaque index, Sulcus bleeding index, Probing depth – measured on medial, buccal, distal and palatal/lingual surface; probing depth will be measured with a Hu-Friedy FDF-GDS periodontal probe;
- ➔ Distance between implant shoulder and mucosal margin (mm);
- ➔ Attachment level;
- ➔ Width of KM;
- ➔ Mobility – measured manually;
- ➔ Standardized PA radiographs;

Analyzing all the data, each implant will be classified as successful or failing according the follow criteria (Buser et al., 1990):

1) Absence of such as pain, foreign body sensation and/or dysaesthesia

2) Absence of peri-implant infection with suppuration;

3) Absence of mobility;

4) Absence of persistent peri-implant radiolucency;

5) Possibility for restoration;

4.11 Step-by-step procedures

Pre-surgical appointment → Digital scan; standardized photographs, CBCT, preoperative medication;

Surgery appointment → Blood collection procedure; L-PRF membrane protocol; surgery; sutures; standardized photographs; CBCT; PA radiographs;

Follow-up appointments:

- ➔ 1st Appointment (10 days after surgery) –To remove sutures; standardized photographs; HI; to collect post-operative questionnaires;
- ➔ 2nd Appointment (30 days after surgery) – standardized photographs; HI;
- ➔ 3th Appointment (3 months after surgery) – standardized photographs; HI; digital scan;
- ➔ 4th Appointment (6 months after surgery) - standardized photographs; PA radiographs; CBCT; digital scan;
- ➔ 5th Appointment (1 year after surgery) - standardized photographs; PA radiographs; success assessment;

4.12 Statistical Analysis

The collected data will be introduced in SPSS software (version 22; IBM SPSS Statistics, Chicago, IL). Results will be expressed as mean +/- 95% Confidence Interval of mm for the bone defect, BML and KM.

First, data will be checked for normality in distribution. Paired Student's t-Test and ANOVA repeated or correspondent non-parametric tests will be used in this analysis. Post hoc tests will be used as appropriate, and Alpha will be set at 0.05.

5.Main Considerations

Several studies conducted with L-PRF in different dentistry fields namely, sinus lift augmentation, horizontal and vertical ridge augmentations, periodontal defects, cyst enucleation, healing of extraction wounds, endodontic surgeries, gingival recessions among others, showed that L-PRF has a positive effect on healing for both soft and hard tissue because of its biological properties (Castro et al., 2017a, 2017b). Additionally, favorable effects on postoperative discomfort reduction were often reported when L-PRF was used (Castro et al., 2017a).

Boora and co-workers, in a randomized control study with immediate implant placement and L-PRF regeneration versus natural healing, described that preservation of peri-implant bone is one of the most important factor for successful implant therapy. L-PRF releases various growth factors which are expressed during different phases of tissue healing, improving tissue repair process. Hence it could serve as therapeutic agents to enhance both peri-implant soft and hard tissue repair. Boora observed a low mean marginal bone in PRF group and 100% dental implant success rate for the one-stage implant placement and immediate provisionalization procedure, after three months. Nevertheless, the author refers that further RCTs with long-term follow-up are needed in order to assess the beneficial effect of L-PRF (Boora et al., 2015).

Oncu et al., in a split-mouth study, designed to evaluate implant stability and recovery using L-PRF, concluded that L-PRF application increased the stability of implants during the first month of healing. The mean marginal bone resorption was 0.7 ± 0.5 mm for the test group and 1.3 ± 0.6 mm for the control group after at least 1 year in function (Öncü & Erbeyoğlu, 2017).

A histologic animal study for wound healing and vertical bone regeneration assessment, concluded that although vertical bone gain had successful outcome with L-PRF at 8 weeks, the highest results were observed when PRF was used in combination with MinerOss® (Potres, Deshpande, Klöeppel, Voss, & Klineberg, 2016). Neiva et al., in an animal study, concluded that higher degrees of bone area fraction occupancy were observed for sockets filled with L-PRF, which may be due to improved cell migration through the stable L-PRF scaffold present between implant and socket walls. Through

histomorphologic observations, it was suggested that L-PRF acts as an efficient barrier during healing (Neiva et al., 2016a).

Simonpieri *et al*. noticed thickening of keratinized gingival tissues that eventually enhanced the esthetic integration and final result of their prosthesis. Furthermore, all their clinical experiences stood out that the use of L-PRF seemed to reduce postoperative edema and pain, and lead to less chances of infectious processes, keeping in mind that L-PRF acts like a healing biomaterial and not a "miracle" product. (Agrawal, 2017; Alain Simonpieri et al., 2009).

A recent clinical study that evaluated L-PRF's effect on socket preservation, showed its efficacy in promoting local soft tissue healing of gums and reducing postoperative pain response, although its alveolar bone response was not significant. The author referred the need for further research comprising large samples to obtain conclusive results (Zhang et al., 2018).

The purpose of this prospective split-mouth study protocol is to evaluate the L-PRF effect on socket healing with immediate implants compared with xenograft.

A split-mouth study design has the advantage to reduce bias concerning inter-individual variability and diminish risk of selection bias since the patient is part of both experimental groups. Although one of the potential disadvantages of this type of study design is carry-across effect, this effect can be excluded, taking into account that the treatments do not have influence on each other. In contrast, the post-operative pain evaluation may have a carry-across effect and the results must be analyzed with caution. Another disadvantage of this kind of study design are its recruitment restrictions, given the fact that selected patients must fulfil every inclusion criteria, and so they may difficult to find (Lesaffre, Philstrom, Needleman, & Worthington, 2009; Needleman, Giedrys-Leeper, Tucker, & Worthington, 2012).

All of the variables chosen to evaluate clinical and radiologic L-PRF effects on socket healing with immediate implants were previously described on the literature. The main purpose of this study is to verify if L-PRF is efficient on socket regeneration and if it can produce such as good results as xenograft, capitalizing its advantages of being less expensive and an autologous material.

In addition to the objective assessment made by clinicians, nowadays there is an increasing focus on patient subjective evaluation (Hartlev et al., 2014). However, patients

seem to be less critical when compared to professionals, and prioritize a short healing time against prosthetic restoration (Hof et al., 2015). Moreover, subjective assessment about post-extraction pain is important to understand if using a different material will improve patient life-quality during the healing process.

Even though L-PRF application indicates good results, systematic reviews revealed that there is a limited number of studies and a lack of standardization regarding this subject, with small samples and short follow ups, thus exposing the need for further RCTs assessing the effect of L-PRF on bone and soft tissue regeneration (Castro et al., 2017b, 2017a; Miron et al., 2017).

This protocol was developed as an attempt to complement current literature regarding the bone and tissue regeneration associated with the L-PRF membrane in immediate implant placement.

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7.Appendix

7.1 Appendix 1 – Informed Consent

Consentimento de Participante em Estudo Clínico

Identificação

Investigador: Natielle Gonçalves
Estudante de 5ºano do Mestrado Integrado de Medicina Dentária
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Orientadores: Professor Doutor João Caramês e Professora Doutora Helena Francisco

PARTE I – Informação

Venho convidá-lo(a) a participar num estudo intitulado "The influence of L-PRF in socket healing with immediate implants: prospective randomized split-mouth study", (A influência do L-PRF na cicatrização alveolar durante a colocação de implantes imediatos: estudo prospetivo randomizado do tipo split-mouth), destinado a homens e mulheres que tenham indicação para colocação de implantes imediatos. Este estudo será realizado tanto com participantes que sejam tratados na Faculdade de Medicina Dentária da Universidade de Lisboa como no Instituto de Implantologia® (consultório privado).

Em caso de dúvida, estamos à disposição para esclarecer e poderá falar com qualquer pessoa acerca da participação neste projeto.

Sempre que se extrai um dente, existe perda óssea que é normal acontecer após extração e cicatrização. Esta perda óssea leva a resultados estéticos e funcionais menos favoráveis. Hoje em dia, existem alguns métodos que ajudam a diminuir a perda de osso e melhorar os tecidos que estão à volta do implante. Um destes métodos é aquele que irá ser investigado neste estudo e irá ser comparado com outro método que já é utilizado há mais tempo que é a utilização de xenoenxerto (enxerto de origem animal). O método a ser comparado com o xenoexerto consiste na produção de uma membrana que ajuda a manter o osso necessário para o bom funcionamento do implante. Esta membrana é feita através do sangue do próprio paciente e colocada no dia da extração.

Assim sendo, os participantes que entrarem neste estudo, terão que tirar sangue (como se tira sangue para análises) no dia da extração do dente para formar a membrana que será colocada no espaço onde estava o dente.

Esta membrana que está a ser investigada é feita 100% através do sangue do participante e não acarreta aumento de risco para a saúde.

Os participantes convidados a participar neste estudo têm que ter mais de 18 anos, serem saudáveis e com indicação para extração do dente e reabilitação com implante imediato.

A participação neste estudo é inteiramente voluntária e a desistência poderá ser efetuada em qualquer fase do estudo, sem qualquer tipo de prejuízo.

Procedimento e Protocolo:

Sabe-se que esta membrana que está a ser testada ajuda a diminuir a perda óssea que acontece após a extração dentária assim como garante um melhor pós-operatório. No

entanto, não está completamente comprovado se essa melhoria é superior à que acontece com a utilização de xenoenxerto. De modo a testar a eficácia desta membrana é necessário testá-la num grupo de participantes com necessidade de, pelo menos, duas extrações sendo que num dos locais da extração será aplicada a membrana e noutro xenoenxerto. Esta escolha é feita de forma aleatória através de um programa informático.

Os investigadores que irão avaliar os resultados, não terão conhecimento acerca de qual dos locais onde foram usadas as membranas, para garantir que não sejam influenciados.

Durante este estudo, serão necessárias pelo menos 6 visitas ao consultório:

- ➔ 1º consulta: extração e colocação do impante, CBCT
- ➔ 2º consulta (após 10 dias): remoção de pontos
- ➔ 3º consulta (após 30 dias): consulta de controlo
- ➔ 4º consulta (após 3 meses): consulta de controlo
- ➔ 5º consulta (após 6 meses): consulta de controlo, CBCT
- ➔ 6ºconsulta (após 1 ano): consulta de controlo

Descrição do procedimento:

Na 1ª consulta será realizada a extração do dente e colocado a sutura. Para os locais de intervenção, que são aqueles em que será aplicada a membrana, será recolhido sangue do braço antes da extração. Após a extração será a colocação de implantes, seguindo todas as normas recomendadas e aprovadas. A membrana será colocada no local onde estava o dente, junto ao implante. Para as localizações de controlo será colocado o xenoenxerto junto ao implante. O que será avaliado é a eficácia da membrana de L-PRF no local onde é colocada, comparando com o local com xenoenxerto.

Na 2ª consulta, serão removidos os pontos, verificada a cicatrização dos tecidos e entrega do questionário.

A 3ª e 4ª consulta serão consultas de controlo, onde será analisada a cicatrização dos tecidos.

Na 5º consulta será apenas para controlar o estado do osso através de realização de radiografia extra e intra-oral.

A dor/desconforto que surgir é a dor habitual da própria extração.

Riscos:

A utilização de membranas para regeneração óssea não tem quaisquer riscos associados, com exceção de uma possível nódoa negra no braço onde é retirado o sangue (equimose).

Benefícios:

Ao participar neste estudo, o participante não só estará a contribuir para encontrar uma resposta ao estudo que está a ser desenvolvido como é um elemento chave. Se forem comprovados os efeitos positivos desta membrana, o participante irá beneficiar de uma melhor cicatrização e de um melhor pós-operatório, constituindo assim vantagens. Tanto o custo de produção da membrana como o exame radiológico que o participante realizar não terá qualquer custo.

Confidencialidade:

Não serão divulgadas quaisquer informações, relativas à identidade dos participantes, a pessoas externas a este estudo. Os dados dos participantes serão

guardados e apenas os investigadores terão acesso. Qualquer informação acerca do participante é referida com um número em substituição do nome.

Publicações dos resultados:

As conclusões retiradas deste estudo serão publicadas perante a comunidade científica. A identidade do participante não será partilhada.

PARTE II – Certificado de Consentimento

Li todas as informações anteriores, ou foram-me lidas. Tive a oportunidade de fazer perguntas acerca do estudo e estas foram respondidas, para minha satisfação. Aceito voluntariamente participar nesta pesquisa.

Nome do Participante_____

Assinatura do Participante _____

Data _____ (Dia /mês/ ano)

Declaração do investigador:

Li com precisão a folha de informações para o participante em potencial e, da melhor forma, certifiquei-me que entenda o que será feito neste estudo.

Confirmo que o participante teve a oportunidade de fazer perguntas sobre o estudo, e todas as perguntas feitas foram respondidas corretamente e na melhor das minhas possibilidades

Confirmo que o participante não foi forçado a dar consentimento, e o consentimento foi dado de forma livre e esclarecida.

Uma cópia deste documento foi fornecida ao participante.

Nome _____

Assinatura _____

Data _____ (Dia/ mês/ ano)

7.2 Appendix 2 - Ethic Committee approval

FACULDADE DE MEDICINA DENTÁRIA
Comissão de Ética para a Saúde (CES-FMDUL)

PARECER

A Comissão de Ética para a Saúde da Faculdade de Medicina Dentária da Universidade de Lisboa (CES-FMDUL), apreciou o pedido de parecer para a realização de um estudo intitulado ***"The influence of L-PRF in socket healing with immediate implants: prospective randomized split-mouth study",*** submetido por Natielle Gonçalves, estudante do 5º ano do Mestrado Integrado de Medicina Dentária, e tendo como orientadores os Professores Doutores João Caramês e Helena Francisco.

A CES-FMDUL deliberou e decidiu emitir **parecer favorável**.

Lisboa, 21 de junho de 2018

O presidente da CES-FMDUL

(Professor Catedrático João Aquino)

7.3 Appendix 3 – McGill pain questionnaire

Versão curta do questionário de dor McGiil :
(Versão Brasileira validada)

Por favor, leia cada palavra abaixo e decida se descreve a dor que sente. Se a palavra não descrever a sua dor assinale "Nenhuma", caso contrário escolha entre as opções "leve", "moderada" ou "severa":

	Nenhuma	Leve	Moderada	Severa
1. Latejante	0	1	2	3
2. Em fiscadas	0	1	2	3
3. Em fincada	0	1	2	3
4. Aguda	0	1	2	3
5. Cólica	0	1	2	3
6. Pressionante	0	1	2	3
7. Em queimação	0	1	2	3
8. Dolorida	0	1	2	3
9. Pesada	0	1	2	3
10. Dolorida à palpação	0	1	2	3
11. Cortante	0	1	2	3
12. Cansativa - Exaustiva	0	1	2	3
13. Nauseante	0	1	2	3
14. Amedrontadora	0	1	2	3
15. Cruel - Punitiva	0	1	2	3

Por favor, marque na escala como, no geral, sua dor se apresentou nos **últimos dias**.

Nenhuma Dor _____ Pior Dor Possível

Qual a intensidade da sua dor ***agora***?

0 Sem dor _____
1 Leve _____
2 Desconfortante _____
3 Angustiante _____
4 Horrível _____
5 Excruciante _____